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The Stereospecific Incorporation of Ornithine into the Tropine Moiety of Hyoscyamine[1]

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The radioactive hyoscyamine obtained when DL-ornithine-2-C^{14} was fed to *Datura stramonium* plants was degraded systematically and found to be labeled entirely on one of the bridgehead carbons of the tropine moiety of the alkaloid.

It has been previously demonstrated[2] that the administration of ornithine-2-C^{14} to *Datura stramonium* plants leads to the formation of radioactive hyoscyamine (I). Degradation established that the activity was located on one or both of the bridgehead carbons (C_1 and C_5) of the tropine moiety of this ester alkaloid. It was of considerable interest to determine whether the incorporation of the ornithine-2-C^{14} was asymmetric affording tropine labeled at only one of the bridgehead carbons.[3] Such a result would favor the direct participation of ornithine in the biosynthesis of tropine and render improbable the suggestion of Wenkert[4] that erythrose is involved.

Differentiation between the two bridgehead carbons of tropine was not possible in our earlier work[2] since the degradative scheme which we used led to the formation of a symmetrical intermediate in which the carbon atoms originally at C_1 and C_5 became equivalent. However, Bothner-By and coworkers[5] devised an ingenious scheme for the degradation of hyoscyamine which permits one to determine whether one or both bridge head carbons are labeled. We have used their degradative scheme with some modifications on radioactive hyoscyamine derived from DL-ornithine-2-C^{14}.

In the present work the tracer was fed to 3-month old *D. stramonium* plants by a wick arrangement.[6] Both the hyoscyamine and the concomitant hyoscine (II) were radioactive. In our previous investigation[2] when ornithine-2-C^{14} was fed to mature (5-month old) plants only the hyoscyamine was active. It has been established[7] that hyoscine is formed from hyoscyamine in various *Datura* species. Our present results thus suggest that hyoscine biosynthesis only occurs in young *D. stramonium* plants. Our studies on the biogenesis of the tropic acid moiety of these alkaloids[8,9] substantiates this view.

Figure 1 illustrates the degradative scheme which was used. Discussion of this scheme is simplified if it is assumed that the bridgehead C_1 is labeled.

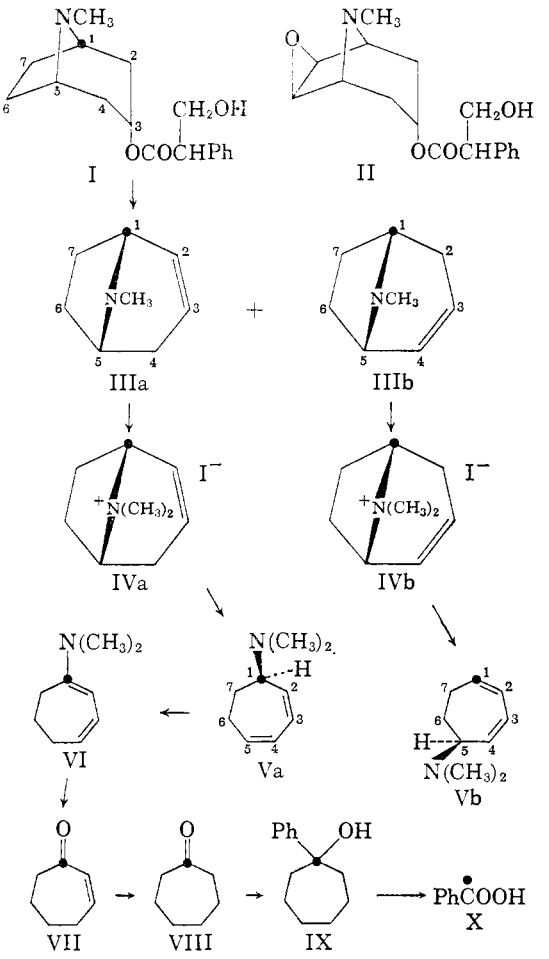

Fig. 1.—Degradative scheme for the radioactive hyoscyamine.

Radioactive carbon is indicated by a heavy dot. Pyrolysis of hyoscyamine in a partial vacuum yielded the isomeric tropidines IIIa and IIIb, which were converted to their methiodides IVa and IVb without separation. Hofmann elimination then gave the enantiomorphic α-methyltropidines Va and Vb which were resolved with dibenzoyl-*d*-tartaric acid. Heating the isomer Va to 160° afforded β-methyltropidine (VI),[10] which was hydrolyzed with dilute sulfuric acid to 2-cycloheptanone (VII). Catalytic hydrogenation yielded cycloheptanone (VIII) which gave 1-phenylcycloheptanol (IX) on treatment with phenyllithium. Oxidation of this alcohol with permanganate gave benzoic acid (X). It was found that the benzoic acid had the same specific activity as radioactive

(1) This investigation was supported by a research grant MY-2662 from the National Institute of Mental Health, Public Health Service.

(2) E. Leete, L. Marion and I. D. Spenser, *Can. J. Chem.*, **32**, 1116 (1954).

(3) In his review on the biogenesis of the tropane alkaloids, G. Fodor ("The Alkaloids," Ed. by R. H. F. Manske, Academic Press, Inc., New York, N. Y., 1960, p. 172.) incorrectly interpreted our results[2] and stated that ornithine-2-C^{14} yielded hyoscyamine labeled at C_1.

(4) E. Wenkert, *Experientia*, **15**, 165 (1959).

(5) A. A. Bothner-By, R. S. Schutz, R. F. Dawson and M. L. Solt, *J. Am. Chem. Soc.*, **84**, 52 (1962).

(6) C. L. Comar, "Radioisotopes in Biology and Agriculture," McGraw-Hill Book Co., Inc., New York, N. Y., 1955, p. 151.

(7) A. Romeike and G. Fodor, *Tetrahedron Letters*, **No. 22**, 1 (1960), and ref. cited therein.

(8) E. Leete, *J. Am. Chem. Soc.*, **82**, 612 (1960).

(9) E. Leete and M. L. Louden, *Chemistry & Industry*, 1405 (1961).

(10) G. Merling, *Ber.*, **24**, 3108 (1891).

TABLE I

	Specific activity, d.p.m./mM. $\times 10^{-6}$
Hyoscyamine hydrochloride	4.0
Tropidine methiodide	3.7
(+)-α-Methyltropidine dibenzoyl-*d*-tartrate	3.8
Cycloheptanone 2,4-dinitrophenylhydrazone	3.9
Benzoic acid	3.8

hyoscyamine (Table I). This of course indicates that all the activity in the hyoscyamine was located at one of the bridgehead carbons. At this time we are unable to specify which of the bridgehead carbons is the labeled one, since we do not know whether the (+)-α-methyltropidine used in the final steps of the degradation had conformation Va or Vb.

Our results are complimentary with those of Bothner-By and co-workers[5] who degraded the radioactive hyoscyamine derived from sodium acetate-1-C^{14}. They found that the carbonyl carbon of the cycloheptanone formed from the radioactive (+)-α-methyltropidine was inactive. This is to be expected from our knowledge of the biosynthesis of ornithine from acetate *via* the Krebs cycle. It has been shown experimentally[11] that the feeding of acetate-1-C^{14} to wheat plants yielded glutamic acid-1,5-C^{14}. This labeled amino acid would then afford ornithine-1,5-C^{14} by accepted biochemical transformations. Acetate-1-C^{14} and ornithine-2-C^{14} thus yield tropines which are labeled at opposite bridgehead positions.

The stereospecific incorporation of *racemic* ornithine-2-C^{14} is remarkable. The plant may of course be only utilizing one of the ornithine isomers, presumably the L-isomer. Furthermore D-ornithine could be converted to L-ornithine *via* α-ketoglutaric acid. It will be of interest to compare the degree of incorporation of radioactive D- and L-ornithine into tropine.

Experimental[12]

Administration of DL-Ornithine-2-C^{14} to *D. stramonium* Plants and Isolation of the Alkaloids.—DL-Ornithine-2-C^{14} monohydrochloride[13] (0.10 mc., 17.3 mg.) was divided equally between twenty 3-month old *D. stramonium* plants growing in soil in a greenhouse (May, 1961).[14] The tracer, dissolved in sterile water, was administered by means of a cotton wick which passed through the stem of the plant about 2 cm. above the soil level. This is now our preferred method of introducing tracers into plants since all the radioactive compound is absorbed by the stems in a few hours and contamination by microörganisms is minimal. After 2 weeks the plants were harvested (fresh wt. 414 g.) and mascerated in a Waring blendor with a mixture of chloroform (500 ml.) and ether (500 ml.), the solution being kept cool by the addition of Dry Ice. Ammonia solution (15 *N*, 50 ml.) was added and the mixture stored with occasional shaking for 2 days. The mixture was then filtered and the two phases separated. The aqueous layer contained 5.3% of the radioactivity which was fed to the plant. The chloroform–ether layer was concentrated to 200 ml. and the alkaloids isolated, separated, and purified by previously described methods.[8] The specific activities of the hyoscyamine hydrochloride (24.3 mg.) and hyoscine hydrochloride (19.2 mg.) isolated without dilution were 4.0×10^6 and 3.0×10^6 d.p.m./mM., respectively.

Degradation of the Hyoscyamine. (a) **Tropidine Methiodide.**—The radioactive hyoscyamine hydrochloride was diluted with inactive alkaloid to give a total wt. of 2.0 g. Pyrolysis of the free base at 280–300° at a pressure of 80 mm. yielded tropidine which was dissolved in ethyl acetate (30 ml.) and treated with methyl iodide (2 ml.). After 16 hr. the copious white precipitate of tropidine methiodide (1.52 g., 93%) was filtered off. A sample for radioactive assay was recrystallized from hot water, small colorless prisms being obtained, m.p. 310–311° dec.

(b) **(+)-α-Methyltropidine Dibenzoyl-*d*-tartrate.**—The active tropidine methiodide (1.5 g.) was dissolved in hot water (15 ml.) and moist silver oxide, formed from silver nitrate (1.5 g.), added and the mixture stirred at 60° for 15 min. The mixture was then filtered and the filtrate evaporated to dryness *in vacuo* at room temperature. The residue was then heated to 120° at a pressure of 0.001 mm., all volatile vapors being condensed in a Dry Ice–acetone trap. Dibenzoyl-*d*-tartaric acid[15] (2.0 g.) dissolved in ethanol (5 ml.) was added to the contents of the trap and the whole evaporated to dryness *in vacuo*. The residue was dissolved in warm ethyl acetate (20 ml.) and on standing colorless prisms of (+)-α-methyltropidine dibenzoyl-*d*-tartrate separated (0.74 g.), m.p. 144–145°. Recrystallization was carried out by dissolving the salt in a small amount of ethanol and then adding about 20 ml. of ethyl acetate. The melting point of this salt is dependent on the rate of heating. A decomposition point of 132–133° was observed if the temperature was raised very slowly. This m.p. agrees with that reported by Bothner-By, *et al.*[5] If the rate of heating was rapid a m.p. of 150–151° with some decomposition was observed. Further recrystallization did not change the rotation of this salt, $[\alpha]^{25}D$ −66° (ethanol).

Anal. Calcd. for $C_9H_{15}N \cdot C_{18}H_{14}O_8$: C, 65.44; H, 5.90; N, 2.83. Found: C, 65.30; H, 5.97; N, 2.85.

The free base had a positive rotation, in agreement with Bothner-By.[5] The picrate of (+)-α-methyltropidine was obtained as yellow plates from ethanol, m.p. 155–156°.

Anal. Calcd. for $C_9H_{15}N \cdot C_6H_3N_3O_7$: C, 49.18; H, 4.95; N, 15.29. Found: C, 49.36; H, 5.05; N, 15.07.

(c) **Cycloheptanone.**—(+)-α-Methyltropidine dibenzoyl-*d*-tartrate (0.68 g.) was dissolved in water, basified with sodium hydroxide, and the solution extracted with chloroform. The dried extract was evaporated and the residual pale yellow oil heated on a metal-bath at 150°. The temperature was raised to 160° and maintained at this temperature for 15 min. The reaction flask was then cooled and 2 *N* sulfuric acid (50 ml.) added and the mixture steam distilled. When 80 ml. of distillate had been collected 10% palladium-on-charcoal (20 mg.) was added and the mixture hydrogenated overnight at a pressure of 30 lb. per square inch. The catalyst was then filtered off and washed well with ether. Evaporation of the ether extract of the filtrate yielded cycloheptanone (23 mg.). A portion was converted to its 2,4-dinitrophenylhydrazone, m.p. 148–149° (yellow plates from ethanol) for radioactive assay.

(d) **1-Phenylcycloheptanol.**—The radioactive cycloheptanone was diluted to give a total weight of 100 mg., dissolved in ether (3 ml.), and added to a cooled solution of phenyllithium prepared from bromobenzene (0.3 ml.), lithium (28 mg.) and ether (2 ml.). The mixture was then stirred at 20° for 3 hr., water was added and the mixture extracted with ether. The dried ether extract was evaporated and the residue dissolved in petroleum ether (b.p. 60–70°) and chromatographed on Florisil (60–200 mesh). Elution with petroleum ether yielded unreacted bromobenzene, then a small amount of diphenyl. No significant material was eluted with 10% benzene in petroleum ether. Pure benzene eluted 1-phenylcycloheptanol (60 mg.) as

(11) E. Bilinski and W. B. McConnell, *Can. J. Biochem. Physiol.*, **35**, 357, 365 (1957).

(12) All melting points are corrected. Microanalyses were determined by Mrs. Olga Hamerston and her assistants at the University of Minnesota. Radioactivity measurements were carried out in a Nuclear-Chicago model C-115 low background Q gas flow counter. Determinations were carried out on samples of finite thickness, making corrections for efficiency and self absorption.

(13) Purchased from Tracerlab, Inc., Waltham, Mass.

(14) The author thanks Mr. Robert C. McLeester of the Botany Department of the University of Minnesota for the cultivation of the *Datura* plants.

(15) C. L. Butler and L. H. Cretcher, *J. Am. Chem. Soc.*, **55**, 2605 (1933).

a colorless viscous oil, whose infrared spectrum (OH absorption at 3350 cm.$^{-1}$) was identical with authentic material.[16]

(e) **Benzoic Acid.**—The radioactive 1-phenylcycloheptanol (56 mg.) was refluxed with potassium permanganate (210 mg.) in water (20 ml.) for 16 hr. The solution was filtered and the acidified filtrate extracted with ether. The dried ether extract was evaporated and the residue sublimed *in vacuo*. The white sublimate was crystallized from hot water yielding colorless plates of benzoic acid (6.3 mg.), m.p. 121–122°, not depressed on admixture with an authentic specimen.

The specific activities of the degradation products of the hyoscyamine are recorded in Table I, corrected for the various dilutions which were made in the course of the degradation.

(16) R. D. Kleene, *J. Am. Chem. Soc.*, **63**, 1482 (1941).

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The Plant Sulfolipid. VI. Configuration of the Glycerol Moiety[1]

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The D-configuration of the glycerol in 6-sulfo-α-D-quinovopyranosyl-(1→1)-glycerol (I) derived from the plant sulfolipid has been demonstrated by radiochemical techniques. The same configuration was observed in the glycerol moieties of β-D-galactopyranosyl-(1→1)-glycerol (IIa) and α-D-galactopyranosyl-(1→6)-β-D-galactopyranosyl-(1→1)-glycerol (IIb) obtained from the galactosyl diglycerides. C^{14}-Labeled glycosyl-(1→1)-glycerols were oxidized with nitrogen dioxide. Glyceric acid-C^{14} was isolated by two-dimensional paper chromatography of acid hydrolysates of the oxidation products. It was co-crystallized with salts of D-, L- and DL-glyceric acids. In each case the specific activity of the L-glyceric acid salt was undiminished by recrystallization. The major glycolipids in plants, therefore, are glycosyl-(1→1′)-2′,3′-diacyl-D-glycerols.

The major lipids of chloroplasts are the galactosyl diglycerides.[4,5] The configuration of the glycerol moiety in these compounds is indicative of specificities involved in phytosynthesis of triglycerides. The most abundant, O-β-D-galactopyranosyl-(1 → 1)-glycerol and O-α-D-galactopyranosyl-(1 → 6)-O-β-D-galactopyranosyl-(1 → 1)-glycerol were first obtained by Carter, McCluer and Slifer,[4] by deacylation of wheat flour lipids. The third most abundant glycolipid in photosynthetic tissues is a sulfolipid with a structure similar to those of the galactolipids. Its deacylation product has been characterized and shown to be 6-sulfo-O-α-D-quinovopyranosyl-(1 → 1)-glycerol (I).[6] These glycolipids are formed concurrently[7] and it is of interest to consider the nature of the diglyceride pools involved in their biosynthesis. The small rotatory contribution of an asymmetric glycerol in these compounds precludes assignment of its configuration based upon rotations of the glycosides.

The availability of C^{14}-labeled glycosylglycerols by deacylation of the lipid products of photosynthesis[7] in $C^{14}O_2$ has made possible a re-evaluation of these structural relationships. This paper reports results of comparison of the configuration of the glycerol moieties in these compounds with those of the authentic asymmetric glyceric acids.

Discussion

O-β-Galactopyranosyl-(1 → 1)-glycerol (G–Gal) (IIa) and O-α-D-galactopyranosyl-(1 → 6)-O-β-D-galactopyranosyl-(1 → 1)-glycerol (G-Gal-Gal) (IIb) were used for model experiments to establish the method. Carter, *et al.*,[4] determined the structure of the G-Gal (m.p. 139–140°, $[\alpha]_D$ +3.77°) and G-Gal-Gal (m.p. 182–184°, $[\alpha]_D$ +86.4°) obtained from wheat flour lipids, except for the configuration of glycerol moiety. G-Gal-Gal (m.p. 196–198°, $[\alpha]^{23}_D$ +88°) was isolated later by Wickberg[8] from the red algae *Polysiphonia fastigiata* and *Corallina officinalis*. It was hydrolyzed to G-Gal with α-galactosidase from a yeast hexokinase preparation. Wickberg synthesized β-D-galactopyranosyl-(1 → 1)-D- (m.p. 140.5–141.5°, $[\alpha]^{20}_D$ −7°) (IIa) and-L-glycerol (m.p. 97–100°, $[\alpha]^{18}_D$ +1°) and concluded from mixed melting point measurements[9] that the naturally occurring G-Gal and G-Gal-Gal possess the D-glycerol structure, although the optical rotation of the natural G-Gal suggested, with less certainty, an L-glycerol structure.

Radiochemical analysis of C^{14}-labeled G-Gal and G-Gal-Gal carried out in this Laboratory has confirmed Wickberg's conclusions. G-Gal obtained by two-dimensional paper chromatography of C^{14}-labeled *Chlorella pyrenoidosa* lipid hydrolysates was oxidized with nitrogen dioxide in dry carbon tetrachloride to a mixture of nitrites of III which was then hydrolyzed with hydrochloric acid and chromatographed to obtain C^{14}-labeled glyceric acid in 36% yield. The small yield (7.3% of the calculated value) of glycolic acid probably was formed by decarboxylation of hydroxymalonic acid, an oxidation product of glycerol. Repeated recrystallizations of authentic asymmetric glyceric acid salts were carried out in the presence of the C^{14}-

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(4) H. E. Carter, R. H. McCluer and E. D. Slifer, *J. Am. Chem. Soc.*, **78**, 3735 (1956).

(5) J. F. G. M. Wintermans, *Biochim. et Biophys. Acta*, **44**, 49 (1960).

(6) H. Daniel, M. Miyano, R. O. Mumma, T. Yagi, M. Lepage, I. Shibuya and A. A. Benson, *J. Am. Chem. Soc.*, **83**, 1765 (1961).

(7) R. A. Ferrari and A. A. Benson, *Arch. Biochem. Biophys.*, **93**, 185 (1961).

(8) B. Wickberg, *Acta Chem. Scand.*, **12**, 1183 (1958).

(9) B. Wickberg, *ibid.*, **12**, 1187 (1958).